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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER KILS117129		
U.S. APPLICATION NO. (if known see 37 C.F.R. 1.5) 09/786992		
INTERNATIONAL APPLICATION NO. PCT/US99/07720	INTERNATIONAL FILING DATE 10 September 1999	PRIORITY DATE CLAIMED 10 September 1998
TITLE OF INVENTION METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE		
APPLICANT(S) FOR DO/EO/US Andreas Gerardus UITTERLINDEN, Petrus Thomas Maria VAN LEEUWEN and Huibert Adriaan Pieter POLS		

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information by Express Mail:

X 1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

____ 2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 37 U.S.C. 371.

X 3. This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).

X 4. The U.S. has been elected by the expiration of 19 months from the priority date (PCT Article 31).

X 5. A copy of the International Application as filed (35 U.S.C. 371(c)(2))

- X a. is attached hereto (required only if not transmitted by the International Bureau).
- ____ b. has been transmitted by the International Bureau.
- ____ c. is not required, as the application was filed in the United States Receiving Office (RO/US).

____ 6. An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).

X 7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

____ a. are attached hereto (required only if not communicated by the International Bureau).

____ b. have been transmitted by the International Bureau.

____ c. have not been made; however, the time limit for making such amendments has NOT expired.

X d. have not been made and will not be made.

____ 8. An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

____ 9. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

____ 10. An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

____ 11. An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

____ 12. An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

____ 13a. A FIRST preliminary amendment.

____ 13b. A SECOND or SUBSEQUENT preliminary amendment.

____ 14. A substitute specification.

____ 15. A change of power of attorney and/or address letter.

X 16. Other items or information:

X a. amended page 2 of the specification and amended claims filed under PCT Article 34; and

X b. copy of the International Preliminary Examination Report.

X ____ 17. The following fees are submitted:.				**CALCULATIONS** PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... $860					
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$710					
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... $690					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100					
ENTER APPROPRIATE BASIC FEE AMOUNT =				$860	
Surcharge of $130 for furnishing the oath or declaration later than ____ 20 ____ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20- 20 =	0	X $18	$	
Independent claims	3 - 3 =	0	X $80	$	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ $270	$	
TOTAL OF ABOVE CALCULATIONS =				$	
_____ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
SUBTOTAL =				$860	
Processing fee of $130 for furnishing the English translation later than ____ 20 ____ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$0	
TOTAL NATIONAL FEE =				$860	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property +				$	
TOTAL FEES ENCLOSED =				$860	
				Amount to be: refunded	$
				charged	$

X ____ 17a. A check in the amount of $860.00 to cover the above fees is enclosed. Check No. 126041.

X 17c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 03-1740. A duplicate copy of this sheet is enclosed.

SEND ALL CORRESPONDENCE TO:

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Respectfully submitted,

CHRISTENSEN O'CONNOR
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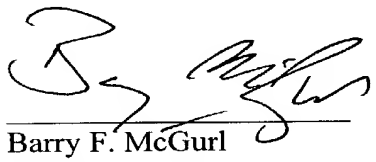

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EXPRESS MAIL CERTIFICATE

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I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

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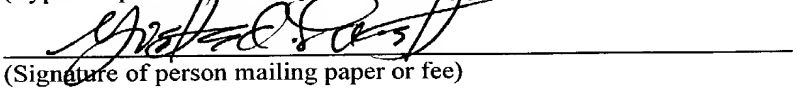

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: A.G. Uitterlinden et al. Attorney Docket No.: KILS117129

Application No.: 09/786,992

Filed: March 9, 2001

Title: METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE

PRELIMINARY AMENDMENT

Seattle, Washington 98101

May 24, 2001

TO THE COMMISSIONER FOR PATENTS:

Please enter the following Preliminary Amendment into the above-referenced patent application.

In the Specification:

On page 1, immediately after the title, please enter the following:

RELATED APPLICATIONS

The present application is the U.S. national phase of PCT/EP99/07720, filed September 10, 1999, which claims benefit of priority from British Patent Application No. GB9819764.3, filed on September 10, 1998, the benefit of priority of which applications is claimed under 35 U.S.C. § 119 and 120.

Amend page 8, lines 1-2 as follows:

1. 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' (SEQ ID NO:1) and/or

2. 5'GCAACTCCTCATGGCTGAGGTCTC-3' (SEQ ID NO:2)

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In The Claims:

Amend Claims 4, 5, 7, 9, 11, 13-16, 18 and 19 as follows.

1. A method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the *Bsm*I, *Apa*I or *Taq*I sites of the vitamin D receptor gene is/are present, wherein the b, a or T allele(s) are associated with risk of heart disease.

2. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine the haplotype of the *Bsm*I, *Apa*I or *Taq*I alleles of the vitamin D receptor.

3. A method according to claim 2 wherein the haplotype may be determined by amplification of a relevant portion of the vitamin D receptor gene, followed by restriction enzyme digestion.

4. (Amended) A method of determining susceptibility to heart disease according to Claim 1, 2 or 3, said method comprising determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor.

5. (Amended) A method according to Claim 1 further comprising determining whether the allele(s) present is/are associated with risk of heart disease.

6. A method according to claim 5 comprising comparing the allele(s) present in the genetic material of the subject with those known to be associated allele(s) of vitamin D receptor genotypes having known ° of risk of heart disease.

7. (Amended) A method according to Claim 1 wherein said method further comprises determining aspects of calcium metabolism in a subject.

8. A method according to claim 7, wherein daily calcium intake of a subject is measured.

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9. (Amended) A method according to Claim 1, wherein said method is performed *in vitro*.

10. A method according to claim 9 wherein said method is performed on blood or tissue samples of a subject.

11. (Amended) A method according to Claim 1 wherein the subject is a mammal.

12. A method according to claim 11 wherein the subject is a human.

13. (Amended) A method according to Claim 11 or Claim 12 wherein the subject is male.

14. (Amended) A method according to Claim 1 for determining susceptibility of a subject to atrial or ventricular hypertrophy, aortic calcification, myocardial infarction, or hypertension.

15. (Amended) A method according to Claim 1 further comprising treating the subject to reduce the risk of heart disease.

16. (Amended) A method according to claim 15 wherein said treatment is selected from the group of treatments consisting of modifications to lifestyle, regular exercise, changes in diet and pharmaceutical preparations.

17. A method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t allele(s) of the vitamin D receptor gene is/are present, in order to determine the underlying cause of the heart disease.

18. (Amended) A method according to claim 17 wherein said subject is first diagnosed as being susceptible to heart disease in accordance with Claim 1.

19. (Amended) A method according to Claims 17 or 18 further comprising administering the appropriate treatment to the subject.

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20. Use of a kit to determine susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) of said gene is/are present.

21. A kit for determining susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) of said gene is/are present; and (iii) means for indicating correlation between said allele(s) and risk of heart disease.

22. A kit according to claim 21, said kit comprising DNA control samples, for comparison with DNA sequences of a subject.

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REMARKS

This preliminary amendment conforms the claim dependencies of the above-referenced patent application to U.S. practice. The Examiner is requested to enter the foregoing amendments prior to examining the application.

Enclosed is a certified copy of the following application for which a claim of priority under 35 U.S.C. § 119 has been made:

Country	Serial No.	Filed
Great Britain	GB9819764.3	10 September 1998

Respectfully submitted,

CHRISTENSEN O'CONNOR
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Barry F. McGurl
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Direct Dial No. 206.695.1775

I hereby certify that this correspondence is being deposited with the U.S. Postal Service in a sealed envelope as first class mail with postage thereon fully prepaid and addressed to the Commissioner for Patents, Washington, D.C. 20231, on the below date.

Date: 5/24/01

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VERSION WITH MARKINGS TO SHOW CHANGES MADE MAY 24, 2001

In the Specification:

On page 1, immediately after the title, the specification has been amended as follows:

RELATED APPLICATIONS

The present application is the U.S. national phase of PCT/EP99/07720, filed September 10, 1999, which claims benefit of priority from British Patent Application No. GB9819764.3, filed on September 10, 1998, the benefit of priority of which applications is claimed under 35 U.S.C. § 119 and 120.

On page 8, lines 1-2 have been amended as follows:

1. 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' (SEQ ID NO:1) and/or

2. 5'GCAACTCCTCATGGCTGAGGTCTC-3' (SEQ ID NO:2)

In the Claims

4. (Amended) A method of determining susceptibility to heart disease according to [claims 1 to 3] Claim 1, 2 or 3, said method comprising determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor.

5. (Amended) A method according to [any one of the preceding claims] Claim 1 further comprising determining whether the allele(s) present is/are associated with risk of heart disease.

7. (Amended) A method according to [any one of the previous claims] Claim 1 wherein said method further comprises determining aspects of calcium metabolism in a subject.

9. (Amended) A method according to [any one of the preceding claims] Claim 1, wherein said method is performed *in vitro*.

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11. (Amended) A method according to [any one of the preceding claims] Claim 1 wherein the subject is a mammal.

13. (Amended) A method according to claim[s] 11 or Claim 12 wherein the subject is male.

14. (Amended) A method according to [any one of the previous claims] Claim 1 for determining susceptibility of a subject to atrial or ventricular hypertrophy, aortic calcification, myocardial infarction, or hypertension.

15. (Amended) A method according to [any one of the preceding claims] Claim 1 further comprising treating the subject to reduce the risk of heart disease.

16. (Amended) A method according to claim 15 wherein [suitable] said treatment[s may include] is selected from the group of treatments consisting of modifications to lifestyle, regular exercise, changes in diet [or] and pharmaceutical preparations.

18. (Amended) A method according to claim 17 wherein said subject is first diagnosed as being susceptible to heart disease in accordance with [any one of] claim[s] 1 [to 16].

19. (Amended) A method according to claims 17 or 18 further comprising administering the appropriate treatment to the subject.

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METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE

The present invention relates to a prognostic method and prognostic means based on polymorphisms in the vitamin D receptor gene. In particular, the present invention relates to a method for determining susceptibility to heart disease by screening for polymorphisms in the vitamin D receptor gene.

Heart disease such as myocardial infarction is a complex phenotype caused by interaction of a number of genetic and environmental factors.[1] Advances in molecular genetics have led to the identification of a number of genetic risk factors for heart disease, such as gene variants involved in thrombosis[2,3] and lipid metabolism[4]. However, another important risk factor for heart disease such as myocardial infarction is a deregulated calcium homeostasis which is required for maintenance of neuromuscular activity[5]. Disturbances of the calcium balance have been implicated in hypertension, ventricular hypertrophy, aortic calcification and arrythmias all of which are important risk factors associated with myocardial infarction.

Vitamin D is a potent regulator of bone and calcium homeostasis, as well as of cellular differentiation and replication in many tissues, and mediates its effects through the vitamin D receptor (VDR). Cloning of the vitamin D receptor has shown it to be a member of the ligand-activated superfamily, which are natural regulators of a number of physiological and developmental processes. The major physiological function of the active metabolite of vitamin D, i.e. $1,25(OH)_2D_3$, is to maintain serum calcium levels in the normal range. This is accomplished by stimulating the small intestine to increase it's efficiency of absorbing calcium from the diet and to mobilize calcium stores from bone. The action of the hormone is mediated by the vitamin D receptor (VDR), a steroid transcription factor that

mediates hormone response by initiating gene transcription and mRNA translation[6]. Involvement of the vitamin D endocrine system in the etiology of heart disease was suggested by epidemiological studies which found serum levels of 25-hydroxyvitamin D_3 to be reduced in myocardial infarction patients compared with control individuals[7]. Furthermore, congestive heart failure associated with vitamin D deficiency has been described in a three and a half month old child[8]. The involvement of the vitamin D endocrine system in cardiac arrythmias was suggested by case reports involving the observation of atrial flutter in a foetus with X-linked vitamin D resistant rickets[9] and sick sinus syndrome that was cured by administration of vitamin D to a 77-year old caucasian woman[10]. Further studies in the rat have also demonstrated an important role of the vitamin D endocrine system in cardiovascular function, wherein vitamin D3-deficiency can lead to large increases in the contractile function of the heart [17-20].

The above studies establish a link between the vitamin D endocrine system and risk of heart disease in an individual. Further studies (Carling *et al*, JCE&M, 82 (6) 1772-1775 (1997); Carling *et al*, Nature Medicine, 1 (12) 1309-1310 (1995) and Carling *et al*, JCE&M 83 (7) 2255-2259 (1998) show an association between the *b*, *a* and *T* alleles of the vitamin D receptor gene and hyperparathyroidism. Hypertension is considered to be a feature of hyperparathyroidism (Boucher, B. J, of Nutr., 79 315-327 (1998). However, the genetic component of heart disease such as myocardial infarction or cardiac arrythmia is poorly defined. As a result, methods of diagnosing risk of heart disease, or treating those suffering from heart disease, are restricted.

Thus, in a first aspect of the present invention, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor is/are present.

The vitamin D receptor gene (12q12) comprises inherited polymorphisms between exon 7 and the 3' UTR of the VDR gene, as shown in Figure 1. These alleles are

denoted B/b, A/a and T/t for restriction enzyme sites *Bsm*I, *Apa*I and *Taq*I respectively (or enzymatic or chemical procedures with similar specificities), where a lower case letter denotes the presence of a wild type restriction site which is capable of being cleaved, and a capital letter denotes the presence of a mutant restriction enzyme site which is not capable of being cleaved by the relevant restriction enzyme. For the purposes of the present invention, determination of which alleles are present in a particular gene may be referred to as determining the genotype of a subject for a particular gene. It is apparent from the above that each copy of the vitamin D receptor gene will comprise a specific combination of the three alleles, this combination being referred to as the haplotype of the gene. For example, the haplotype may be baT, indicating the presence of cleavable *Bsm*I and *Apa*I sites, and a non-cleavable *Taq*I site. Direct haplotyping of the VDR gene has allowed five different haplotypes to be determined, of which three are common.[16]

The present invention is based on the discovery of a genetic component of heart disease. It has now been shown that presence of the b, a or T alleles, and in particular the baT haplotype of the vitamin D receptor is/are associated with increased risk of heart disease. Thus, the presence of such alleles of the vitamin D receptor gene may be used to determine susceptibility to heart disease.

Preferably, the method of the first aspect of the present invention comprises the additional step of determining whether the alleles present are associated with risk of heart disease. This may be performed by comparing the alleles present in the genetic material of the subject with genotypes of the vitamin D receptor having known degrees of risk of heart disease. For example, a visual aid detailing alleles and their relative risk of heart disease may be used to determine whether the genotype of the subject is associated with a high or low risk of heart disease.

The first aspect of the present invention may also comprise the additional step of determining aspects of calcium metabolism, such as calcium levels, in a subject. Preferably, the daily calcium intake is measured. This feature of the first aspect is based on the observation that the correlation between vitamin D receptor genotype and heart disease may be dependent upon dietary calcium intake.

The method of the first aspect may be performed *in vitro*. Preferably, the method is performed on tissue or fluid removed from the body of the subject. Thus, the present invention relates to a non-invasive method, the results of which provide an indication of susceptibility to heart disease but do not lead to a diagnosis upon which an immediate medical decision regarding treatment has to be made.

The present invention may be performed on any subject for whom it is desirable to determine risk of heart disease. Preferably, the subject may be a mammal. Most preferably, the method of the first aspect is performed on humans, preferably male.

Heart disease may include atrial or ventricular hypertrophy, aortic calcification, arrythmia, hypertension, and most importantly, myocardial infarction.

In a preferred feature of the first aspect of the present invention, there is provided a method of determining susceptibility to heart disease, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the vitamin D receptor gene is/are present. The method may comprise determining whether one or more of the above alleles is present. The subject may further be classified as being heterozygous or homozygous for one or more alleles. Preferably, the method comprises the additional step of determining whether the allele(s) present is/are associated with risk of heart disease, wherein presence of the

b, a or T alleles is associated with increased risk of heart disease. Homozygosity for the a, b or T allele may further increase the susceptibility to heart disease in a subject.

In a preferred feature of the first aspect, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising analysing the genetic material of a subject to determine the haplotype of the *Bsm*I, *Apa*I and *Taq*I alleles at the vitamin D receptor. Preferably, said method comprises determining whether the haplotype of the subject is associated with risk of heart disease, wherein the haplotype baT is associated with high risk of heart disease. A subject homozygous for said haplotype may be at a higher risk of heart disease than those heterozygous for the haplotype.

In a preferred feature of the first aspect, there is provided a method of determining susceptibility to heart disease, said method comprising the additional step of determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor, where an increase in copy number is associated with increased risk of heart disease.

The present invention may be performed using any suitable method known in the art. Preferably, a tissue or fluid sample is first removed from a subject. Examples of suitable samples include blood, mouth or cheek cells, and hair samples containing roots. Other suitable samples would be known to the person skilled in the art. The genetic material is then extracted from the sample for diagnosis, using any suitable method. The genetic material may be DNA or RNA, although preferably DNA is used. For example, the DNA may be extracted using the technique described in Sambrook *et al* (Molecular Cloning- A Laboratory Manual, Cold Spring Harbor Laboratory Press). Determination of the genotype of a subject may then be carried

out using the extracted DNA, employing any one of the following techniques:

- Southern blot analysis following digestion with one or more appropriate restriction enzymes.
- PCR amplification followed by digestion with one or more appropriate restriction enzymes and, optionally, separation of digestion products by gel electrophoresis.
- Sequencing of a relevant gene fragment by any suitable method.
- Visualization of heteroduplex patterns, for example on PAA or agarose gels, where different patterns may indicate the presence of one or more specific alleles.
- Separation of DNA fragments using denaturing gradient gels, wherein the degree of separation will depend upon the presence or absence of one or more polymorphic restriction sites.
- Separation using SSCP analysis, the patterns of which will depend upon the presence or absence of one or more polymorphic restriction sites.
- Use of allele specific oligonucleotides, hybridization patterns of which will be specific for various combinations of alleles.
- Methods such as OLA, Taqman or dot-blot for the detection of known mutations.
- Visualization of DNA sites using fluorescent labelled probes for alleles of interest.
- RFLP analysis

Where it is desirable to use particular restriction enzymes in performing the present invention, the skilled person will understand that enzymatic or chemical procedures having similar specificities may also be used. For example, restriction enzymes having similar specificity (isoschizomers) to those described herein may be used, or chemical degradation procedures with DNA or RNA cutting specificity.

Other techniques suitable for determining the genotype of a subject may be used in the present invention.

Where the haplotype of a gene is to be determined, it is preferable to use a direct haplotyping method, as described in Uitterlinden *et al*[16]. In such a method, the relevant portion of the gene is amplified and then subjected to restriction enzyme digestion, in order to determine the presence or absence of restriction enzyme sites. Thus, for example, where the haplotype of the vitamin D receptor gene is to be determined, the portion of the gene between exon 7 and the 3' UTR may be amplified, and the amplified DNA digested with the *Bsm*I, *Apa*I or *Taq*I restriction enzymes. Gel analysis may then be used to determine which alleles are present.

Preferably, a fragment may be amplified using polymerase chain reaction (PCR) techniques, to produce copies which, where the fragment is of the vitamin D receptor, are at least 1000 base pairs in length, and most preferably at least 1800 base pairs in length. PCR techniques are well known in the art, and it is within the ambit of the skilled person to identify primers for amplification of the appropriate region of the above genes, namely the region from exon 7 to the 3' UTR of the vitamin D receptor gene. PCR techniques are described in EP-A-0200362 and EP-A-0201184.

In a preferred feature of the first aspect, there is provided a method of determining susceptibility to heart disease in a subject, said method comprising amplifying a fragment comprising a portion of the region from exon 7 to the 3' UTR of the vitamin D receptor gene, and determining which allele(s) in the vitamin D receptor is/are present. Primers suitable for amplification of said portion of the vitamin D receptor gene would be readily available to a person skilled in the art. Examples of such primers include:

1. 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and/or
2. 5'-GCAACTCCTCATGGCTGAGGTCTC-3'

Where the amplified portion of the gene is larger than the above defined portion of the vitamin D receptor gene containing the relevant alleles, it is preferable to avoid the inclusion of gene sequences which comprise any one of the *Bsm*I, *Apa*I or *Taq*I restriction sites.

In a second aspect of the present invention, there is provided a method of therapy, said method comprising treating a subject diagnosed as being at risk of heart disease, to reduce the risk of heart disease. Preferably, the subject is diagnosed as being at risk of heart disease in accordance with the first aspect of the present invention.

Therapy may in the form of preventative or palliative care. Suitable treatments include modifications to lifestyle, regular exercise and changes in diet. Suitable treatments, including pharmaceutical preparations, would be known to physicians and persons skilled in the art. Examples include ACE inhibitors, beta-blockers, calcium or vitamin D preparations, magnesium sulphate, thrombolytics and analgeasics.

In a third aspect of the present invention, there is provided a method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which allele(s) of the vitamin D receptor gene is/are present. Preferably, the method includes first determining whether the subject is susceptible to heart disease. Where a subject has been determined as susceptible to heart disease, the method may further comprise administering the appropriate treatment. The present aspect of the invention is based on the observation that agents, such as calcium channel blockers, which are useful for treatment of a variety of cardiovascular diseases may also be associated with cardiovascular morbidity in some cases. The effect of an

agent may therefore depend on the underlying cause of the heart disease. For example, the presence of the b, a or T alleles of the vitamin D receptor may result in modification of calcium uptake, leading to impaired cardiovascular function. Thus, in such a case it would be preferable to avoid the use of calcium channel blocking agents.

In a fourth aspect of the present invention, there is provided use of a kit to determine which allele(s) of the vitamin D receptor gene is/are present, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) is/are present in said gene.

Preferably, the primer molecules are suitable for amplification of at least a portion of the region between exon 7 and the 3'UTR of the vitamin D receptor gene. Examples of suitable primers are described above.

Means for determining which allele(s) is/are present in the vitamin D receptor gene, may include any reagents or molecules necessary for use in any of the methods described above. For example, where PCR followed by DNA digestion is used, said means preferably include PCR reagents and one or more of the *Bsm*I, *Apa*I, or *Taq*I restriction enzymes. Where the method employs Southern Blotting, heteroduplex visualization, or fluorescent labelling techniques for example, probes which bind to the appropriate regions of the vitamin D receptor gene may be included. Where necessary, such probes may be labelled to allow detection, for example by nick-translation, radio- or fluorescent-labelling, or random primer extension whereby the non-labelled nucleotides serve as a template for the synthesis of labelled molecules. Other methods of labelling probes are well known in the art.

In a preferred feature of the fourth aspect of the present invention, there is provided use of a kit further comprising means for indicating correlation between the genotype of a subject and risk of heart disease. Said means may be in the form of a chart or

visual aid, which indicate that presence of the b, a or T alleles or baT haplotype of the vitamin D receptor is/are associated with heart disease such as cardiac arrythmia or myocardial infarction.

In a fifth aspect of the present invention, there is provided a kit for determining risk of heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) is/are present in said gene; and (iii) means for indicating correlation between the allele(s) and risk of heart disease.

In a preferred feature of the fifth aspect, the kit may also comprise DNA control samples, for comparison with DNA sequences of a subject. The control samples may comprise the sequence of one or more alleles of the vitamin D receptor gene, or may comprise the sequence of various haplotypes.

Preferred features of each aspect of the present invention are as for each other aspect, mutatis mutandis.

The present invention will now be described in detail with reference to the following examples and figures in which:

FIGURE 1 is a schematic presentation of the region between exon 7 and the 3' UTR of the vitamin D receptor gene.

Example 1 - Relationship between alleles of the vitamin D receptor gene and myocardial infarction, cardiac arrythmias and dietary calcium intake in the elderly

METHODS

Study Subjects

The Rotterdam Study is a population-based cohort study of 7983 subjects aged 55 or more years, residing in the Ommoord district of the city of Rotterdam in the Netherlands. The study was designed to document the occurrence of disease in the elderly in relation to several potential determinants.[15] A total of 10,275 persons, of whom 9161 (89 percent) were living independently, were invited to participate in the study in 1991. In the independently living subjects, the overall response rate was 77 percent for home interview and 71 percent for examination in a research centre, including measurement of anthropometric characteristics and blood sampling. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus University Medical School and written informed consent was obtained from each subject.

The analysis of the association between *VDR* genotype, myocardial infarction and arrytmias was performed in a subgroup of men and women participating in the study. Baseline measurements, collected from 1990-1993, included and electrocardiogram (ECG), history of cardiovascular disease, established cardiovascular risk factors, and use of medication. A digitally stored ECG was available for 5931 independently living subjects from the study, but 1453 of these were excluded on the basis of age (>80 yrs), use of a walking aid, diabetes mellitus or use of diuretic, estrogen, thyroid hormone or cytostatic drug therapy. From the 4478 remaining subjects, we studied a random sample of 2000 men and women aged 55 to 80 years. ECG data or DNA samples were not available for 22 subjects, resulting in a final study group of 1978 subjects.

Measurements

Height and weight were measured at the initial examination in a standing position without shoes. Myocardial infarction, including so called silent myocardial infarctions, was confirmed by ECG analysis. For 1725 men and women (87%) data

was available on dietary intakes of calcium (mg/day) during the preceding year. These were assessed by food frequency questionnaire and adjusted for energy intake. Age at menopause and current cigarette smoking were assessed by questionnaire. Cardiac arrythmias were diagnosed by ECG analysis and included the occurrence of sinus irregularities, atrial flutter, and atrial fibrillation and were recorded, confirmed and classified by a physician. For 976 subjects data on cardiac arrythmias were available (49%). Of these subjects, data on dietary calcium intake were available for 899 subjects (92%).

Determination of VDR Genotypes

Genomic DNA was extracted from peripheral venous blood samples according to standard procedures and the anonymous polymorphisms were detected by PCR as previously described[16]. Three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, i.e. for BsmI, ApaI, and TaqI, were assessed in relation to each other by a direct molecular haplotyping PCR procedure which we developed[16]. This allows us to determine ophase of the alleles at each of the RFLP loci and as a result three frequent haplotype alleles are discerned, encoded 1 (baT; frequency 48%), 2 (BAt; frequency 40%), 3 (bAT; frequency 10%) combining to six genotypes encoded 11, 12, 13, 22, 23, and 33. Detailed information on haplotype alleles and genotype frequencies in the Rotterdam Study can be found elsewhere[16]. The PCR reaction mixture of 25 microlitres contained 100 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dideoxynucleotidetriphosphates, 150 ng of each primer, and 0.2 unit of Super Taq polymerase (HT Biotechnology, Cambridge, UK). The reactions were performed in a DNA thermocycler (model 480, Perkin Elmer) with a cycling protocol of 94°C, 60°C, and 72°C for 1 min each, for 28 cycles. Ten microlitres of PCR product were simultaneously digested with 5 units BsamI, 5 units ApaI, and 5 units TaqI restriction enzyme and 2 microlitres of a 10x concentrated buffer

(containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 35 mM $MgCl_2$) by incubating for 30 min at 37°C, followed by a 30 min incubation at 65°C. The digestion products were analysed on a 1.2 percent agarose gel run in 44.5 mM Tris, 44.5 mM boric acid, 1 mM Na_2EDTA) for 300 volt-hours. Separation patterns were documented by Polaroid photography under UV-illumination (302 nm). To confirm the accuracy of genotyping, repeat analysis was performed on 200 random samples. No discrepancies were found.

Statistical Analysis

Relevant clinical variables were compared between the genotype groups by analysis of covariance and multiple linear regression was used to adjust values for confounding factors such as age, anthropometric variables, smoking. For the comparisons we made reference, heterozygote and homozygote groups for each of the VDR alleles. For example, for VDR allele1 the groups comprised 22, 23, and 33 for the reference group, 12, and 13 for the heterozygote group, and 11 for the homozygote group. The Chi-squared test was used to test for genotype distribution in men and women with and without myocardial infarction and with and without cardic arrythmias. Odds ratios (with 95 percent confidence interval) were calculated by multivariate logistic regression analysis to estimate the relative risk of myocardial infarction and cardiac arrythmias. A multivariate regression model was designed to test for the influence of dietary calcium intake on the relationship between VDR genotype and myocardial infarction and VDR genotype with cardiac arrythmias. For this analysis, subjects were categorised into groups of calcium intake defined by quartiles of the population. An interaction term was included in the regression analysis defined as gene allele dose multiplied with calcium-intake. P values for all statistical tests were two-sided.

RESULTS

Characteristics of the Study Subjects

Anthropometric, and dietary measurements in the men and women with the six VDR genotypes are shown in Table 1. The allele frequencies (1=48.6%; 2=40.6%; and 3=10.8%) and the distribution of genotypes were similar to that reported in a previous study[22]. There was no difference with regard to age, dietary calcium intake, smoking habits, body mass index, serum HDL-cholesterol , and serum cholesterol in the six genotype groups.

Association with Myocardial Infarction

The distribution of myocardial infarction by VDR genotype in men and women is shown in Table 2. There was modest over-representation of both men and women carrying the VDR haplotype allele 1 as compared with subjects without the allele although this did not reach significance. Logistic regression analysis showed that subjects in the heterozygous group had a 1.2 fold increased risk for myocardial infarction and the subjects in the homozygous group had a 1.5-fold increased risk, as compared with subjects not carrying the VDR allele 1. The gene dose effect was 1.2-fold increased risk per copy of the VDR allele 1. The gene dose effect was larger for women when compared to men (RRs were 1.1 (95% confidence interval 0.9 - 1.4) for men and 1.5 (95% confidence interval 1.1 - 2.2) for women) but the interaction term of sex times genotype in the regression model was not significant (P=0.65). The relative risks for myocardial infarction did not change after adjustment for potential confounding factors such as age, body mass index and smoking in the regression analysis. For VDR haplotype allele 2 and haplotype allele 3 no significant differences were observed (data not shown).

Modification of the Association by Dietary Calcium Intake

When the study subjects were stratified according to quartiles of dietary calcium intake, the differences in distribution of myocardial infarction by VDR genotype were restricted to individuals with the highest intake of calcium (1302 mg/day or more). These differences corresponded with 5.4-fold increased risk for heterozygote carriers and 8.3 -fold increased risk for homozygote carriers of VDR haplotype allele 1. Considering age, genotype and myocardial infarction together in a multivariate regression model, we found a significant modifying effect of dietary calcium intake on the VDR genotype effect on risk for myocardial infarction ($P=0.04$).

Association with Cardiac Arrythmias

We subsequently analysed the distribution of cardiac arrythmias by VDR genotype. We did not observe differences in the distribution of arrytmias by VDR genotype in the total study group. However, in the group with high calcium intake (1302 mg/day or more) there was significant over-representation of cardiac arrythmias for subjects carrying the VDR haplotype allele 1 as compared with subjects without the allele 1 (Table 4).

Logistic regression analysis showed that subjects in the heterozygous group had a 1.6 fold increased risk for cardiac arrythmias and the subjects in the homozygous group had a 3.6-fold increased risk, as compared with subjects not carrying the VDR allele 1. The relative risks for cardiac arrythmia did not change after adjustment for potential confounding factors such as age, body mass index and smoking in the regression analysis. Also when myocardial infarction was entered into the model the relative risks did not change.

DISCUSSION

Polymorphisms of the vitamin D receptor gene are associated with the risk of myocardial infarction. The increased risk was especially notable in subjects with a high dietary calcium intake. In this group, we also observed VDR haplotype 1 to be associated with increased risk of cardiac arrythmias. Cardiac arrythmias have been frequently observed following a myocardial infarct event. However, the VDR genotype dependent risk for cardiac arrythmias we observe, is independent of myocardial infarction. This indicates that the genotype effect we observe for myocardial infarction is not mediated by the genotype effect on cardiac arrythmias.

Our results indicate a genetic susceptibility of the risk for myocardial infarction and cardiac arrythmias in subjects with a relatively high dietary calcium intake. This strongly suggest that the VDR genotype effect on myocardial infarction and cardiac arrythmias is mediated by an effect on calcium transport. Treatment with calcium and vitamin D has been indicated for arrythmias and hypertrophy and has been shown to improve myocardial function.

The hormone vitamin D has been shown to influence the Ca-flux in a variety of cell types including heart muscle cells. These rapid effects of the hormone are suggested to involve a non-genomic pathway by which the plasmalemmal VDR is coupled to plasma membrane Ca2+ channel proteins.

Calcium channel blocking agents are indicated for the treatment of a variety of cardiovascular diseases, including cardiac arrythmias, but have also been found associated with increased cardiovascular morbidity[21,22]. Therefore, the modification by calcium intake of the VDR genotype dependent effect on myocardial infarction we observe opens the possibility that genotyping at the VDR gene locus might be of value in predicting the response to treatment with some of these drugs. Prospective

clinical trials are needed to investigate the clinical and therapeutic implications of our results.

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CLAIMS

1. A method of determining susceptibility to heart disease in a subject, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the *Bsm*I, *Apa*I or *Taq*I sites of the vitamin D receptor gene is/are present, wherein the b, a or T allele(s) are associated with risk of heart disease.

2. A method of determining susceptibility to heart disease according to claim 1, said method comprising analysing the genetic material of a subject to determine the haplotype of the *Bsm*I, *Apa*I or *Taq*I alleles of the vitamin D receptor.

3. A method according to claim 2 wherein the haplotype may be determined by amplification of a relevant portion of the vitamin D receptor gene, followed by restriction enzyme digestion.

4. A method of determining susceptibility to heart disease according to claims 1 to 3, said method comprising determining the copy number of the B/b, A/a or T/t alleles or haplotype of the vitamin D receptor.

5. A method according to any one of the preceding claims further comprising determining whether the allele(s) present is/are associated with risk of heart disease.

6. A method according to claim 5 comprising comparing the allele(s) present in the genetic material of the subject with those known to be associated allele(s) of vitamin D receptor genotypes having known degrees of risk of heart disease

7. A method according to any one of the previous claims wherein said method further comprises determining aspects of calcium metabolism in a subject.

8. A method according to claim 7, wherein daily calcium intake of a subject is measured.

9. A method according to any one of the preceding claims, wherein said method is performed *in vitro*.

10. A method according to claim 9 wherein said method is performed on blood or tissue samples of a subject.

11. A method according to any one of the preceding claims wherein the subject is a mammal.

12. A method according to claim 11 wherein the subject is a human.

13. A method according to claims 11 or 12 wherein the subject is male.

14. A method according to any one of the previous claims for determining susceptibility of a subject to atrial or ventricular hypertrophy, aortic calcification, myocardial infarction, or hypertension.

15. A method according to any one of the preceding claims further comprising treating the subject to reduce the risk of heart disease.

16. A method according to claim 15 wherein suitable treatments may include modifications to lifestyle, regular exercise, changes in diet or pharmaceutical preparations.

17. A method of predicting the response of a subject to treatment, said method comprising analysing genetic material of a subject to determine which of the B/b, A/a or T/t allele(s) of the vitamin D receptor gene is/are present, in order to determine the underlying cause of the heart disease.

18. A method according to claim 17 wherein said subject is first diagnosed as being susceptible to heart disease in accordance with any one of claims 1 to 16.

19. A method according to claims 17 or 18 further comprising administering the appropriate treatment.

20. Use of a kit to determine susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene, and (ii) means for determining which allele(s) of said gene is/are present.

21. A kit for determining susceptibility to heart disease in a subject, said kit comprising (i) one or more nucleic acid primer molecules for amplification of a portion of the vitamin D receptor gene; (ii) means for determining which allele(s) of said gene is/are present; and (iii) means for indicating correlation between said allele(s) and risk of heart disease.

22. A kit according to claim 21, said kit comprising DNA control samples, for comparison with DNA sequences of a subject.

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(57) Abstract

The present invention relates to prognostic method and means for determining susceptibility to heart disease in a subject by screening for polymorphisms in the Vitamin D receptor gene. In particular, the present invention provides a method for determining susceptibility to heart disease, the method comprising analysing the genetic material of a subject to determine which of the B/b, A/a or T/t alleles of the restriction enzyme sites *BsmI*, *ApaI* and *TaqI* respectively are present. Specific combinations of alleles represent a haplotype which is associated with susceptibility to heart disease.

TABLE 1.

Characteristics ot the Population According to *VDR* Genotype

CHARACTERISTIC†	*VDR* GENOTYPE						
	11	*12*	*13*	*22*	*23*	*33*	P-VALUE
Number (%)	493 (24.9)	735 (37.2)	202 (10.2)	351 (17.7)	170 (8.6)	27 (1.4)	
Age (years)	67.0 ± 6.8	67.1 ± 6.8	67.2 ± 7.1	67.0 ± 7.1	66.9 ± 6.8	67.0 ± 7.1	0.93§
Body Mass Index (kg/m^2)	26.1 ± 3.7	26.0 ± 3.3	25.8 ± 3.6	26.1 ± 3.4	26.0 ± 3.2	25.5 ± 2.9	0.78
Dietary calcium-intake (mg/day)	1116 ± 350	1122 ± 364	1122 ± 356	1092 ± 369	1094 ± 342	1158 ± 254	0.78
Serum HDL-cholesterol (mmol/l)	1.34 ± 0.37	1.35 ± 0.36	1.36 ± 0.34	1.35 ± 0.37	1.32 ± 0.33	1.36 ± 0.38	0.91
Serum cholesterol (mmol/l)	6.68 ± 1.21	6.63 ± 1.26	6.64 ± 1.16	6.60 ± 1.19	6.59 ± 1.21	6.60 ± 0.96	0.95
Current Smokers (%)	130 (26.4)	172 (23.4)	45 (22.3)	78 (22.2)	40 (23.5)	6 (22.2)	0.83¶

† Values are means ± standard deviation; BMI is weight divided by the square height

§ P-value for ANOVA

¶ P-value for Chi-2 test

TABLE 2.

Myocardial Infarction According to *VDR allele 1* Genotype

	Men		Women		All	
	MI (%)	Total	MI (%)	Total	MI (%)	Total
Total	151 (15.8)	954	62 (6.1)	1024	213 (10.8)	1978
by *VDR allele 1* genotype						
Reference†	39 (14.7)	266	10 (3.5)	282	49 (8.9)	548
Heterozygotes	69 (15.4)	449	31 (6.4)	488	100 (10.7)	937
Homozygotes	43 (18.0)	239	21 (8.3)	254	64 (13.0)	493
χ^2	1.18		5.38		4.43	
P-VALUE	0.55		0.07		0.11	
Odds Ratios for Myocardial Infarct by VDR allele 1 genotype [95% CI]						
Crude						
Reference	1.00		1.00		1.00	
Heterozygotes	1.07 [0.72 - 1.71]		1.86 [0.90 - 3.85]		1.23 [0.86 - 1.76]	
Homozygotes	1.28 [0.80 - 2.05]		2.48 [1.15 - 5.39]		1.53 [1.03 - 2.27]	
per copy *VDR 1* allele	1.13 [0.89 - 1.44]		1.53 [1.07 - 2.20]		1.24 [1.02 - 1.51]	
Age-, BMI-adjusted						
Reference	1.00		1.00		1.00	
Heterozygotes	1.11 [0.72 - 1.71]		1.77 [0.85 - 3.68]		1.22 [0.85 - 1.75]	
Homozygotes	1.33 [0.82 - 2.14]		2.45 [1.12 - 5.34]		1.55 [1.04 - 2.30]	
per copy *VDR 1* allele	1.15 [0.91 - 1.47]		1.53 [1.06 - 2.22]		1.25 [1.02 - 1.52]	

† "Reference" includes VDR genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

TABLE 3.

Myocardial Infarction According to VDR allele 1 Genotype by Quartiles of Dietary Calcium Intake

	< 877 mg/day		> 877, < 1076		> 1076, < 1302		≥ 1302	
	MI (%)	Total	MI (%)	Total	MI (%)	Total	MI (%)	Total
Total	43 (10.0)	432	50 (11.6)	431	45 (10.4)	432	49 (11.4)	430
by *VDR allele 1* genotype								
Reference†	13 (9.9)	131	14 (12.5)	112	12 (9.6)	125	3 (2.6)	114
Heterozygotes	21 (10.5)	200	24 (11.8)	204	21 (10.0)	210	26 (12.6)	207
Homozygotes	9 (8.9)	101	12 (10.4)	115	12 (12.4)	97	20 (18.3)	109
χ^2	0.19		0.25		0.53		14.17	
P-VALUE	0.91		0.88		0.77		0.0008	
Odds Ratios for Myocardial Infarct by VDR allele 1 genotype [95% CI]								
Crude								
Reference	1.00		1.00		1.00		1.00	
Heterozygotes	1.09 [0.52 - 2.27]		0.93 [0.46 - 1.89]		1.04 [0.49 - 2.20]		5.40 [1.59 - 18.3]	
Homozygotes	0.90 [0.37 - 2.20]		0.82 [0.36 - 1.87]		1.32 [0.56 - 3.09]		8.31 [2.39 - 29.0]	

† "Reference" includes VDR genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

TABLE 4.

Myocardial Arrythmias According to *VDR allele 1* Genotype by Quartiles of Dietary Calcium Intake

	< 877 mg/day		> 877, < 1076		> 1076, < 1302		≥ 1302	
	MA (%)	Total	MA (%)	Total	MA (%)	Total	MA (%)	Total
Total	37 (12.1)	307	27 (9.2)	292	17 (5.6)	302	31 (10.1)	306
by *VDR allele 1* genotype								
Reference†	16 (17.0)	94	6 (8.8)	68	7 (7.7)	91	5 (5.7)	88
Heterozygotes	14 (10.1)	138	14 (10.1)	138	6 (4.4)	135	12 (8.5)	141
Homozygotes	7 (9.3)	75	7 (8.1)	86	4 (5.3)	76	14 (18.2)	77
χ^2	3.19		0.27		1.11		7.80	
P-VALUE	0.20		0.87		0.58		0.02	
Odds Ratios for Myocardial arrythmias by VDR allele 1 genotype [95% CI]								
Crude								
Reference	1.00		1.00		1.00		1.00	
Heterozygotes	0.57 [0.26 - 1.23]		1.13 [0.41 - 3.12]		0.54 [0.18 - 1.69]		1.60 [0.54 - 4.74]	
Homozygotes	0.51 [0.20 - 1.32]		0.92 [0.29 - 2.92]		0.69 [0.19 - 2.46]		3.63 [1.22 - 10.9]	

† "Reference" includes VDR genotypes 22, 23, 33; "Heterozygotes" includes 12, 13; "Homozygotes" includes 11

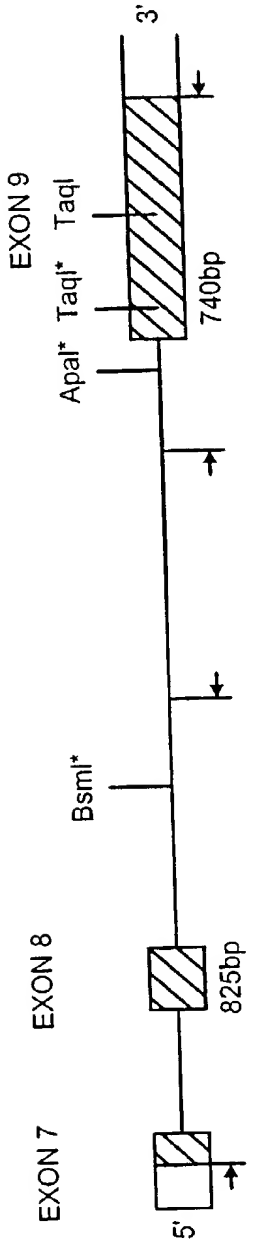

FIG. 1

Attorney Docket No. KILS117129

COMBINED DECLARATION AND POWER OF ATTORNEY
IN PATENT APPLICATION

As a below-named inventor, I hereby declare that:

my residence, post office address, and citizenship are as stated below next to my name;

I believe that I am an original, first, and joint inventor of the subject matter that is claimed and for which patent is sought on the invention entitled METHOD FOR DETERMINING SUSCEPTIBILITY TO HEART DISEASE BY SCREENING POLYMORPHISMS IN THE VITAMIN D RECEPTOR GENE, the specification of which was mailed to the Patent and Trademark Office on March 9, 2001, and assigned United States Patent Application No. 09/786,992.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(c), of any foreign application(s) for patent listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

Number	Country	Day/Month/Year Filed	Priority Claimed Yes/No
9819764.3	Great Britain	10 September 1998	yes

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(d), of any inventor's certificate listed below. I declare that, upon investigation, I am satisfied that to the best of my knowledge, when filing the application for the inventor's certificate I had the option to file an application for either a patent or an inventor's certificate as to the subject matter of the identified claim or claims forming the basis for the claim of priority:

I hereby claim the benefit under Title 35, United States Code, Section 119(e), of any United States provisional application(s) listed below: NONE

I hereby claim the benefit under Title 35, United States Code, Section 120, of any United States application(s) or PCT international application(s) designating the United States listed below, and, insofar as the subject matter of each of the claims of this application is not

disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application: NONE.

Prior PCT Application:

Application No.	Filing Date	Status
PCT/EP99/07720	10 September 1999	abandoned

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith: Bruce E. O'Connor, Reg. No. 24,849; Lee E. Johnson, Reg. No. 22,946; Gary S. Kindness, Reg. No. 22,178; James W. Anable, Reg. No. 26,827; James R. Uhlir, Reg. No. 25,096; Jerald E. Nagae, Reg. No. 29,418; Dennis K. Shelton, Reg. No. 26,997; Jeffrey M. Sakoi, Reg. No. 32,059; Ward Brown, Reg. No. 28,400; Robert J. Carlson, Reg. No. 35,472; Marcia S. Kelbon, Reg. No. 34,358; Rodney C. Tullett, Reg. No. 34,034; Daiva K. Tautvydas, Reg. No. 36,077; Mary L. Culic, Reg. No. 40,574; Julie C. VanDerZanden, Reg. No. 38,105; George E. Renzoni, Ph.D., Reg. No. 37,919; and Philip P. Mann, Reg. No. 30,960; and the firm of Christensen O'Connor Johnson Kindness^{PLLC}. Address all telephone calls to Barry F. McGurl at telephone No. 206.695.1775.

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I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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